AD_____

GRANT NUMBER DAMD17-96-1-6173

TITLE: Potential Role of the Tumor Suppressor ADENOMATOUS POLYPOSIS COLI in Polarization of Breast Epithelial Cells

PRINCIPAL INVESTIGATOR: Dr. Kristi Neufeld

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, UT 84102

REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (15 Jul 96 - 14 Jul 97)

4. TITLE AND SUBTITLE
Potential Role of the Tumor Suppressor ADENOMATOUS POLYPOSIS COLI in Polarization of Breast Epithelial Cells

5. FUNDING NUMBERS
DAMD17-96-1-6173

6. AUTHOR(S)
Dr. Kristi Neufeld

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Utah
Salt Lake City, UT 84102

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Recent evidence suggests that the adenomatous polyposis coli (APC) gene participates in breast tumorigenesis. Although a precise biological function for APC protein has not yet been determined, it has been shown that the APC protein interacts with β-catenin and plakoglobin *in vivo*. β-catenin and plakoglobin are components of two specialized anchoring junctions, the adherens junction, a site of attachment for bundles of actin filaments, and the desmosome, a site of attachment for intermediate filaments (e.g. keratin). A direct correlation has been shown between loss of adherens junction components and the metastatic potential of breast cancer. I have used a combination of immunofluorescence microscopy and biochemical fractionation to determine the location of APC protein in epithelial cells from both normal and breast cancer tissue. APC protein was located in the nucleus and the cytoplasm of all breast epithelial cells tested. APC protein concentrated at the edge of breast epithelial cells was eliminated by disruption of keratin filaments and microtubules, but not by actin disruption. APC protein appeared tightly associated with intermediate filaments of the normal breast epithelial cell following sequential extraction. These findings are consistent with APC protein interacting with intermediate filaments, but not with actin filaments.

14. SUBJECT TERMS Breast Cancer	15. NUMBER OF PAGES
Adenomatous Polyposis Coli, Intermediate Filaments, Anchoring Junction, Microtubules, Actin Filaments	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

KH In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

KH In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

KH In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Kristi L Neufeld 8/13/97
PI - Signature Date

TABLE OF CONTENTS

Section	Page #(s)
Front Cover	1
SF298	2
Foreword	3
Table of Contents	4
Introduction	5-6
Body of Report	
Experimental Methods	7-12
Results	13-20
Discussion and Recommendations in relation to the Statement of Work	21-22
Conclusions	22
References	23-24
Appendix	
Figure 1	25-26
Figure 2	27
Figure 3	28
Figure 4	29
Figure 5	30-31
Figure 6	32-33

INTRODUCTION

Recent evidence suggests that the adenomatous polyposis coli (APC) gene participates in breast tumorigenesis. *APC* gene loss is reported in as many as 28% of human breast tumors (1). Female mice carrying mutant *APC* genes show an increased rate of breast hyperplasia and neoplasia (2). In addition, APC has been linked to the murine proto-oncogene Wnt-1, which was originally identified as a frequent target for insertional activation by mouse mammary tumor virus in mammary carcinomas. In vertebrates, APC protein is proposed to be a component of the Wnt-1 signaling pathway. Together, these data suggest a key role for APC in the breast cancer pathway.

Although a precise biological function for APC protein has not yet been determined, it has been shown that the APC protein interacts with β-catenin and plakoglobin *in vivo* (3, 4, 5). Like APC, β-catenin is a component of the Wnt-1 signaling pathway. In the absence of Wnt-1, the APC protein down-regulates β-catenin. If Wnt-1 is present, APC does not target β-catenin for degradation, thus allowing for it's accumulation in the cytoplasm and subsequent transport to the nucleus where it can effect gene expression. β-catenin and plakoglobin are components of two specialized anchoring junctions. Anchoring junctions mechanically attach epithelial cells (and their cytoskeleton) to neighboring cells or to the extracellular matrix. β-catenin and plakoglobin are found in the adherens junction, a site of attachment for bundles of actin filaments. Plakoglobin is also a component of the desmosome, a site of attachment for intermediate filaments (e.g. keratin). Anchoring junctions also serve an important role in signal transduction, mediating changes in cyto-architecture and proliferation. A direct correlation has been shown between loss of adherens junction components and the metastatic potential of breast cancer.

The purpose of this study is to determine whether APC interacts directly with adherens junctions or desmosomes through actin and keratin respectively. If APC protein can be demonstrated to be a component of this junction then this could explain the link between APC loss and breast cancer.

My research has demonstrated that breast epithelial cells containing only one wild-type copy of the APC gene have a severe deficiency in their ability to establish polarity when compared to normal breast-epithelial control cells. I am presently investigating the hypothesis that the APC protein plays an essential role in normal breast epithelial cell polarity and its absence contributes to the loss of cellular differentiation and growth regulation found in malignant transformation. In the first year of investigation, I have tested cells from human breast-cancer lines, as well as epithelial cell lines established from normal breast tissue for localization and expression of APC protein, using both a biochemical and histological approach. These experiments have led to the investigation of potential interactions between the APC protein and intermediate filaments of the cytoplasm and nucleus.

BODY

EXPERIMENTAL METHODS

Cell lines and Tissue Culture. Cell lines used in these experiments were maintained at 37^{o} C in CO_2 (5%) incubators. 184A1 cells are an immortalized human mammary epithelial cell line (a gift from Martha Stampfer). They were grown in MCDB 170 media (Clonetics Corporation, La Jolla, CA) supplemented as described (6). Other cells were obtained from the American Type Culture Collection (ATCC, Gainsburg, MD) and were maintained in the following growth media: MCF7 [Eagle's Modified Essential Media (MEM), 10% Fetal Bovine Serum (FBS), 1% nonessential amino acids, Earle's Basic Salt Solution, 1 mM sodium pyruvate, 10 ug/ml insulin], MDA-MB468 [Dulbecco's MEM (DME), 10% FBS], BT549 and T47D [RPMI 1640, 10% FBS].

Immunofluorescence microscopy. Cells were seeded onto tissue culture chamber slides (25-50% confluency) and allowed to grow for 36 - 48 h before manipulation. Cells were rinsed in Phosphate Buffered Saline (PBS) [10 mM phosphate, pH 7.5, 100 mM NaCl] then fixed with 2% paraformaldehyde in PBS for 30 minutes (min) at 4^{o} C. Following two PBS rinses, cells were permeabilized with 0.2% tritonX-100 in Tris Buffered Saline (TBS) [10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM KCl] for 5 min at room temperature. Following two TBS washes, cells were incubated with 0.5% Na_2BH_3 in water for 10 min at room temperature. Cells were rinsed with TBS and then incubated with primary antibody diluted in antibody buffer [1% Bovine Serum Albumin (BSA), 3% normal goat sera, 0.2% tritonX-100 in TBS] for 90 min at room temperature. Cells were rinsed three times with TBS prior to incubation with secondary

antibody conjugated to Fluorescein-isothiocyanate FITC, rhodamine or Texas Red for 30 min at room temperature. Cells were rinsed three times with TBS and mounted with Pro Long antifade (Molecular Probes, Eugene, OR) for immunofluorescence microscopy. Antibodies and dilutions used for the experiments are as follows: APC (mouse IgG1, Ab-4, Ab-2, or Ab-6) 1:150 (Oncogene Science, Cambridge, MA), or APC (rabbit, APC64, a gift from Arnold Levine) 1:100; α-tubulin (mouse IgG1, DM-1A) 1:200 (ICN Biomedicals, Inc. Costa Mesa, CA), keratin (guinea pig, K-4252) 1:300 (Sigma Immuno Chemicals, St. Louis, MO), lamin A/C (mouse IgG1, X-67) or lamin B (mouse IgG1, X233) undiluted (American Research Products, Inc., Belmont, MA), goat anti mouse IgG1-FITC and goat anti mouse IgG1-Texas Red 1:200 (Southern Biotechnology Assoc. Inc., Birbingham, AL), goat anti guinea pig-rhodamine 1:400 and goat anti rabbit-Texas Red 1:200 (Accurate Chemical and Scientific Corp, Westbury, NY), and goat anti rabbit-FITC 1:200 (Boehringer Mannheim biochemicals, Indianapolis, IN). APC polyclonal sera was adsorbed to DLD-1 cells that had been fixed and permeabilized as described for immunofluorescence staining. For APC antibody blocking experiments, APC antibody was incubated with a peptide corresponding to APC protein amino acids 2717 - 2844 at 10-fold molar excess in PBS for 12 hours at 4^{o} C. Any precipitant protein was pelleted by centrifugation for 15 min prior to dilution of the peptide/antibody mixture in antibody buffer.

Disruption of Cellular filaments. Three different drug treatments were used to disrupt the microtubule structure: 50 mM colchicine at 37^{o} C for three hours, 50 mM γ-lumicolchicine at 37^{o} C for three hours, 10 mM nocodazole at 37^{o} C for one hour, and 10 mg/ml vinblastine at 37^{o} C for

one hour. Cytochalasin B was used at 10 mM at 37^{o} C for 20 min for actin disruption experiments. Keratin intermediate filaments were disrupted by introduction of Keratin antibody into cells with lysophosphatidylcholine (LPS) as described (7). Control (IgG1) antibodies were introduced in a similar manner as a negative control.

Cell Fractionation. Cell fractionation was performed as described (8). For all cell fractionation or sequential extraction steps, protease inhibitors (Boehringer Manheim, Indianapolis, IN) were added to the buffers in the following concentrations: pefablock (0.2 mg/ml), aprotinin (0.01 mg/ml), pepstatin (0.01 mg/ml) and leupeptin (0.01 mg/ml). Briefly, cells grown in 150-cm^{2} flasks were harvested by scraping into ice cold PBS. Cells were rinsed two times with cold PBS prior to lysis with detergent. Cells were resuspended in L-buffer [PBS, 0.1% tritonX-100 and 0.1% NP-40] and incubated on ice for 10 min. Cells were determined to be >99% lysed using trypan blue exclusion. Nuclei were pelleted by centrifugation at 1000 x g for 10 min at 4^{o} C. Supernatant was further fractionated by centrifugation at 100,000 x g for 60 min, 4^{o} C. The supernatant fraction was collected and classified as cytoplasm. The pellet was resuspended in L-buffer and is considered to be the membrane/cytoskeletal fraction. The nuclear pellet was purified from membrane contaminants by two rinses in L-buffer, passage through a 0.22 gauge needle three times, and passage through a 0.85M sucrose cushion (15,000 rpm, microfuge, 15 min). Nuclei in the pellet were lysed by sonication (30 sec) in L-buffer prior to DNase (100U/200 ml) treatment, 45 min, 4^{o} C. Nuclei were sonicated two times, 30 sec, 4^{o} C to make a nuclear lysate. For nuclear scaffold/matrix isolation, nuclear pellets purified through the sucrose cushion were washed

with Nuclei Buffer [10 mM Tris, pH 7.4, 20 mM KCl, 0.125 mM spermidine, 0.05 mM spermine, 1% thiodiglycol] one time before resuspension in Nuclei Buffer. DNase I (1000U) and $MgCl_2$ (5 mM final) were added and allowed to incubate on ice for 30 min. $CuSO_4$ (100mM final) was added and the nuclei were incubated for 10 min, 37° C. Nuclear scaffold proteins were precipitated on ice by addition of an equal volume of 0.4 M $(NH_4)_2SO_4$ in 10 mM trisHCl, 0.2 mM $MgCl_2$. Precipitate was raised in 15 ml TM-0.2 buffer [10 mM tris HCl, pH 7.4, 0.2 mM $MgCl_2$, 0.2 M $(NH_4)_2SO_4$] and pelleted at 1500 rpm, 15 min, 4° C. The pellet was washed three times with Nuclei Buffer and 70 mM NaCl prior to resuspension in 2X sodium dodecyl sulfat-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [4% SDS, 20% glycerol, 120 mM Tris, pH 6.8]. Protein concentration was determined in all cell fractions using a Bradford Method as per manufacturers instructions (BioRad, Hercules, CA).

Immunoprecipitation. Cells were harvested in cold PBS as described and lysed by sonication (2 x 30 seconds) in L-buffer. Insoluble proteins, membranes, and DNA were removed by centrifugation 15,000 rpm, 15 minutes. Proteins in the supernatant were incubated with 25 ul protein A sepharose beads 120 mg/ml (Sigma, St. Louis, MO) for 60 min, 4°C. Protein A Sepharose was removed along with cellular proteins that bind nonspecifically to it by centrifugation at 6500 RPM for 5 minutes. Primary antibody (2 mg) was incubated with the preadsorbed lysate for 60 minutes at 4°C on a rotator platform. Protein A sepharose (25 ul) was added to the antibody/lysate mixture and allowed to incubate for 1 hour at 4°C. Protein A Sepharose and primary antibody were removed, along with

cellular proteins recognized by the primary antibody by centrifugation at 6500 RPM for 5 minutes. The supernatant from this centrifugation step was saved for SDS-PAGE. The pellet was washed two times with L-buffer and three times with RIPA buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxysholate (DOC), 0.1% SDS]. Final pellets were resuspended in 2X SDS-PAGE sample buffer and boiled for 5 minutes. Protein A Sepharose was removed by centrifugation at 6500 RPM for 5 minutes, prior to loading on a gradient gel for SDS-PAGE.

Sequential Extraction. Sequential fractionation of cells prior to immunofluorescence microscopy was performed essentially as described (9). Briefly, cells grown on chambered glass slides were exposed to the following sequential extraction buffers for 5 min each at 4^{o} C unless otherwise specified. Cells were rinsed in PBS for five min between each step. Cytoskeletal buffer [10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% tritonX-100]. Extraction buffer [10 mM PIPES, pH 6.8, 250 mM $(NH_4)_2SO_4$, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% tritonX-100]. Digestion buffer [10 mM pipes, pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% tritonX-100, DNase (500U), RNase (0.1 mg/ml)] for 30 min 30°C. High salt buffer [10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% tritonX-100, and 2M NaCl]. Following extraction, cells were rinsed with PBS and processed for immunofluorescence microscopy as described.

Western Immunoblot. Proteins (70 µg/lane; 35 µg/lane for scaffold fractions) were separated electrophoretically using 4-12% acrylamide

gradient tris tricine gels (Novex, San Diego, CA) and Laemmli buffer. Gels were run for 2 hr at 125V with cold circulating water. Proteins were transferred to nitrocellulose (Schleicher and Schuel, Keene, NH) for 16 hours at 30V in transfer buffer [192 mM glycine, 20% Methanol, 25 mM tris base and 0.1% SDS] with circulating cold water. Rainbow molecular weight markers (Amersham, Arlington Heights, IL) were loaded in one lane of each gel for size standardization. Nitrocellulose membranes containing transfered proteins were blocked with 5% BSA in TBST [TBS, 0.1% tween 20] then incubated with primary antibody diluted in 0.5% BSA in TBST for 1 hr, 20^{O} C. Following three, 10-min rinses with TBST, blots were incubated with a the appropriate secondary antibody conjugated to HRP in 0.5% BSA/TBST. Blots were rinsed three times with TBST and then probed using ECL chemiluminensence detection system (Amersham) as per manufacturer's instructions. Antibodies used for Western Immunoblot analysis were as follows: APC (mouse IgG1, Ab-1 or Ab-2) 1:500 (Oncogene Science), a-tubulin 1:200, a-adaptin (mouse IgG2a, AP-2) 1:200 (Sigma Immunochemicals), EGF-receptor (rabbit sera, Ab-4) 1:100 (Oncogen Science), lamins A, B, and C 1:10, HRP-Rabbit anti mouse IgG1 1:20,000 (Zymed Immunochemicals, South San Francisco, CA), horse radish peroxidase (HRP)-goat anti rabbit IgG and HRP-sheep anti mouse 1:40,000 (Sigma Immunochemicals),

RESULTS

Location of APC protein in breast epithelial cells from normal and cancer cell lines as determined by imunofluorescence microscopy.

Task 1: Plate cells from breast cancer lines established in the lab (~20) and purchased from ATCC (3, initially) on glass chamber slides. Test cells for wild-type APC protein expression by immunofluorescence analysis. Characterize localization of APC protein in the cancer lines grown on a slide.

Task 10: Sequentially fractionate normal HMECs. Determine APC fractionation profile by Western immunoblot and immunofluorescence microscopy.

The distribution pattern for endogenous, full-length APC protein in various cell lines was determined using indirect immunofluorescence microscopy with antibodies that recognize the C-terminus of APC protein. The staining pattern in four breast cancer cell lines, BT549, MDA-MB468, MCF7 and T47D, was compared to those seen in cells from primary outgrowth of normal breast epithelial tissue (BE20, BE-21) and the "normal" breast cell line 184A1. Because of technical difficulties we have been unable to establish cell lines from any of the seven breast cancer tissue specimens we have received in the past year, therefore, the current studies focus on lines available from ATCC. 184A1 is an immortalized human mammary epithelial cell line that displays keratin staining patterns and phenotypic characteristics reminiscent of normal human mammary epithelial cells (10). These cells express full-length APC protein as determined by Western blot analysis (Fig. 3a, lane T) and were used as representative of normal epithelial cells.

Of seven monoclonal and three polyclonal antibodies tested in immunofluorescence assays with the 184A1 cell line, the three monoclonal antibodies raised against the C-terminus of APC protein (monoclonals Ab-2, Ab-4 and Ab-6), and the polyclonal antibody APC64, also raised against the C-terminus, gave similar and reproducible staining patterns, as seen in

Figure 1b, c, g, h, and i. These antibodies localized full-length APC protein to two distinct compartments of the epithelial cell. A punctate staining pattern is visible in the cytoplasm, with especially prominent staining at cell edges (1b, 1g - i, solid arrows). In migrating cells, the APC staining concentrated at the leading edge (data not shown). This cytoplasmic distribution of APC protein provides additional support for the very similar results reported recently in MDCK cells (11). In addition to the cytoplasmic staining, however, there is significant nuclear staining, shown most clearly in (1c, open arrow), where the same cells viewed in (1a) and (1b) are photographed at a slightly different focal distance. Much of the total staining seems to be concentrated in discrete nuclear regions (open arrows). In (1a), the cells shown in (1b) and (1c) were stained with 4',6-diamidino-2-phenylindole (DAPI) and viewed using Differential Interference Contrast (DIC) optics to distinguish cell nuclei, cell edges, and general morphology. Normal primary epithelial cells that migrated from human breast tissue specimens (BE20 and BE21) had an identical APC distribution pattern as the 184A1 (data not shown). As further proof of the specificity of immunofluorescent signal using the APC antibody, DLD-1 cells were stained using the C-terminal APC antibodies. As expected, since DLD-1 is a colon cancer cell line that expresses no full-length APC protein, these cells give no APC signal (Fig. 1k).

Cells from the four breast cancer lines also showed a similar staining pattern to that seen in 184A1 cells. In each case, cells were stained using antibodies directed against the C-terminus of APC protein (monoclonal Ab-4 and polyclonal sera, APC64) and IgG1 as a negative control. T47D (Fig. 1j) and MDA-MB468 (Fig. 2a) cells displayed both punctate cytoplasmic staining, concentrated at the leading edge, and nuclear staining. A similar pattern was seen in BT549 cells, with additional filamentous staining visible

in the cytoplasm (Fig. 2b). Non-confluent MCF7 cells also displayed the punctate cytoplasmic and nuclear staining pattern (Fig. 2c). However, in areas of higher cell confluency, the staining pattern was strikingly different. While the nuclear staining was still clearly visible, a significant portion of the APC protein appeared to be located at cell-cell junctions (Fig. 2d).

Biochemical fractionation was employed as an independent test of the several cellular locations of APC protein in normal breast epithelial cells. 184A1 cells were subjected to lysis by detergent (>99% cell lysis as determined by trypan blue exclusion) followed by purification of the nuclei from the cytosolic fraction. The cytosolic fraction was further separated into a membrane/cytoskeletal fraction and a soluble, cytoplasmic fraction. Nuclear matrix/scaffold proteins were further purified from the nuclear fraction by DNAse treatment, stabilization with $CuSO_4$, precipitation in 0.2 M ammonium sulfate, and washes. The fractions were verified by Western Immunoblot using antibodies directed against compartment-specific proteins: α-tubulin fractionated with the cytoplasm (Fig. 3b), α-adaptin with membranes (Fig. 3c, lane M/S), and lamins A, B, and C with the nuclear and nuclear matrix/scaffold fractions (Fig. 3d lanes N and Sc).

184A1 cells contained full-length APC protein in both the membrane/cytoskeletal (M/S) and the nuclear (N) fractions. The slowest band recognized by the APC antibody migrated at the molecular size predicted for full-length APC protein relative to the molecular weight markers (Fig. 3a, M/S and Sc) and was recognized by antibodies raised to both N-terminal (Fig 3a) and C-terminal (data not shown) regions of APC protein. Band intensities were greatly diminished by preincubation of the APC antibody with an APC peptide (data not shown).

Western Immunoblot analysis of APC protein in breast cancer cell lines.

Task 2: Test cells found to have no detectable level of APC protein in Task 1 for APC expression by Western immunoblot analysis. Determine percentage of breast cancer lines that have decreased APC protein compared to normal.

In the colon, >99% of the inactivating APC mutations result in production of a truncated APC protein product, thus implying that point mutations are not sufficient to inactivate the very large APC protein. In addition, surveys of colon polyps (colon cancer precursors) show that the vast majority have inactivated both copies of the APC gene (12). Assuming that the same is true for epithelial cells of the breast, it is possible to catalogue the percentage of breast cancer cell lines with fully-inactivated APC by testing these lines for the presence of full-length APC protein.

Because all four breast cancer cell lines showed significant immunofluorescence staining using antibodies directed against the C-terminus of APC, it is likely that they all express full-length APC protein. In order to confirm the presence of full-length APC protein by a second method, cells from two of the four breast cancer lines were tested for APC expression by Western blot analysis. Lysates from both BT549 and MDA-MB468 contained a 320 kDa protein that was recognized by an antibody raised against APC protein's N-terminus (data not shown). Based on the combined immunofluorescence and Western immunoblot data, I conclude that four out of four breast cancer cell lines tested express full-length APC protein.

Creation of "null" breast epithelial cells for use in polarization assay

Task 4: Clone APC antisense construct into retroviral vector. Infect normal HMECs with APC-antisense retroviral construct in order to prevent APC protein expression. Grow infected cells in selection media. Estimate infection efficiency by detection of GFP by

immunofluorescence microscopy. Test APC expression level (Western immunoblot and immunofluorescence).

Since all four breast cancer cell lines tested appear to have normal APC protein, it was necessary to create an APC "null" cell line in order to fully test potential APC functions. Several methods have been employed thus far, each based on the ability of an antisense oligonucleotide to block translation of the APC protein. The first method involved design and synthesis of DNA oligomers, 18 nucleotides long, that corresponded to sequences "antisense" to APC mRNA, either in the 5' untranslated region (APC-A5) or spanning the initiation codon (APC-AC) (Fig. 4a). "Sense" oligos were synthesized in parallel for use as negative controls (APC-S5 and APC-SC). The oligos were purified by HPLC, dialyzed, then added to the growth media of 184A1 cells, both in the presence and absence of a lipofection reagent. Lipofection has been reported to improve permeability of DNA oligos into some types of cells (13). Although a range of oligo concentrations was tested, using two different antisense oligos, I was unable to detect changes in APC protein level as determined by immunofluorescence analysis (data not shown).

For the second antisense approach, the entire APC gene was cloned in reverse orientation into the expression vector pCDNA3.1 (Invitrogen) (Fig. 4b). Cells were co-transfected with the antisense APC construct and a green fluorescent protein- (GFP) expression construct so that APC protein level could be monitored specifically in those cells that were successfully transfected. Although GFP-expressing cells could be identified after co-transfection, no reduction in APC protein level could be detected by immunofluorescence analysis.

The third antisense approach utilizes the retroviral construct pLXSN (Clontech) containing the complete cDNA for APC in reverse orientation

(Fig. 4c). Transfection of a colon cancer cell line that contained no endogenous full-length APC protein with this construct did not result in alteration of cellular morphology. I have yet to test these transfected cells for decreased APC protein level.

Task 5: Introduce APC antibodies into normal HMECs by phospholipid-mediated antibody delivery. Determine antibody transfection efficiency by immunofluorescence microscopy using FITC-conjugated secondary antibody and no primary antibody. Test APC expression level (Western immunoblot and immunofluorescence).

In case the retroviral APC antisense approach fails, or as a complementary approach should it succeed, I have begun to test whether APC function can be eliminated by the introduction of APC antibodies into a cell. To this end, I have dialyzed and concentrated APC monoclonal antibody (Ab-4) and polyclonal sera (APC64), and then introduced them into 184A1 cells using either LPC-mediation or microinjection. At this preliminary stage, since the APC protein has no measurable activity, the endpoint of such experiments has been to look for global changes in cellular morphology and re-distribution of potential APC protein partners (e.g. β-catenin, keratin, tubulin) in the injected cell compared to control cells. The successful introduction of antibodies into cells was demonstrated by staining cells using only a FITC-conjugated secondary antibody.

Test directly for interactions between APC protein and the cytoskeletal components (actin and keratin) of the cell-cell anchoring junctions in <u>normal</u> breast epithelial cells

Task 9: Precipitate keratin and actin proteins from normal HMEC lysate. Test for co-precipitation of APC protein

In order to demonstrate interaction between APC protein and filament proteins keratin and actin, I have tested for co-

immunoprecipitation. These experiments have been performed by precipitation with APC antibodies followed by Western immunoblot with keratin and actin antibodies as well as the converse. Although I was able to demonstrate co-precipitation of APC and β-catenin protein using this method, I was unable to clearly show a specific interaction between APC protein and keratin or actin (data not shown). This might be because keratin and actin are both in large complexes which mask the epitopes recognized by the precipitating antibodies. Similarly, APC antibody might only be able to precipitate soluble forms of APC protein that are in complex with only a few other proteins (such as β-catenin). Alternatively, the interaction might be only transitory or weak.

As the first of two alternative method to test for possible interactions between APC protein and keratin or actin filaments I have chosen to disrupt the filaments in 184A1 cells with either cytochalasin B (for actin) or inactivating antibodies (for keratin) and test for redistribution of the APC protein by immunofluorescence microscopy. Cytochalasin B, which disrupts actin microfilaments, did not alter the distribution of APC protein (Fig. 5d). In contrast, when breast epithelial cells were subjected to keratin antibody treatment which induced collapse of the keratin filaments, APC protein at the leading edge was almost completely lost (data not shown, but similar to Fig. 5a). This treatment has no effect on actin or tubulin organization, suggesting that the APC protein concentrated at the leading edge is dependent on intact keratin filaments. 184A1 cells treated with microtubule depolymerizing agent cochicine (Fig. 5b), as well as with nocodazole and vinblastine (data not shown), two other microtubule depolymerizing agents also showed elimination of the APC staining at the leading edge. γ-lumicolchicine, a derivative of colchicine that does not disrupt microtubules but has the same nonspecific effects as colchicine, had

no affect on APC localization (Fig. 5c). Tubulin, actin and keratin were shown to be redistributed or eliminated by the specific drug or antibody treatments by antibody staining.

As a second alternative approach, immunofluorescence microscopy provided further support for the association of APC protein with the keratin intermediate filament network. Proteins not tightly bound to intermediate filaments were sequentially removed from 184A1 cells, using conventional extraction techniques (9). This extraction procedure removed components not associated with the intermediate filament or nuclear scaffold network, including microtubules, soluble proteins, actin, histone H1, and DNA-binding and RNA-binding proteins. Figure 6 shows an immunofluorescence microscopic analysis of cells either with no treatment (left two columns) or fully extracted (right two columns). APC protein remained cell-associated following complete extraction. Comparing the relative staining intensity of the second and fourth panels and adjusting for differences in exposure rate, it appeared that the APC protein signal increased in intensity following extraction. This increase in staining intensity occurred after the first extraction step which released soluble proteins and tubulin (data not shown), leading to the suggestion that the increase was due to the release of proteins that mask the APC protein signal in untreated cells. Following extraction, APC protein remained associated, not only with the nuclear matrix, but also with the intermediate keratin filaments of the cytosol. In contrast, tubulin, actin, and DNA (compare DIC/DAPI lanes from extracted and unextracted cells) were quantitatively released.

Discussion and Recommendations in relation to the Statement of Work:

Endogenous APC protein has been found in both the nucleus and the cytoplasm of cultured human breast epithelial cells. Immunocytochemistry revealed a particulate distribution of APC protein in distinct nuclear regions, and throughout the cytoplasm, with concentrations at the leading edge of migrating cells. Each of three monoclonal, and one polyclonal, antibodies directed against APC protein's C-terminus consistently showed the same staining pattern. In contrast, five antibodies directed against the APC protein's N-terminus failed to show significant staining, suggesting that the N-terminal epitopes might be masked *in vivo,* or that these antibodies might recognize denatured but not native protein.

Surprisingly, all four breast cancer cell lines tested expressed full-length APC protein. The distribution of APC protein in cells from these lines was remarkably similar to that in normal breast cells. The MCF-7 cell line revealed an additional location for APC protein, the cell-cell junction. In the next year, I plan to further characterize the APC pattern in confluent MCF-7 cells by determining whether the APC protein appearing at the cell-cell junctions is co-localized with known components of the adherens junction β-catenin, E-cadherin, actin or plakoglobin. Cell fractionation experiments provided further support for a nuclear as well as cytoskeletal/membrane localization for the APC protein.

Since none of the breast cancer cell lines tested lacked full-length APC protein, I will acquire and test more lines from ATCC. Also, several approaches are being taken to create an APC-deficient breast epithelial cell. If the antisense methods prove successful, then the polarization studies will

proceed, as planned. Introduction of an inactivating APC antibody to a population of cells *via*. LPC is being explored as well.

Two separate lines of experimentation point to an interaction between APC protein and keratin. Using keratin antibodies to collapse intermediate filaments, I have shown that APC protein distribution is dependent on intact intermediate filaments. Additionally, APC protein remains associated with intermediate filaments following sequential extraction. In the next year this potential interaction will be further explored.

CONCLUSIONS

1. APC protein is located in the nucleus and the cytoplasm of normal breast epithelial cells.

2. Breast cancer cells lines tested (4) show expression of full-length APC protein with a similar distribution pattern as that seen in normal breast epithelial cells.

3. APC protein concentrated at the edge of breast epithelial cells is eliminated by disruption of keratin filaments and microtubules, but not by actin disruption.

4. APC protein appears tightly associated with intermediate filaments of the normal breast epithelial cell following sequential extraction.

REFERENCES

1. Frixen, U. H., et. al., *J. Cell. Biol.* **113**: 173-185 (1991).

2. Gabbert, H., Wagner, R., Moll, R. and Gerharz, C.-D, *Exp Metastasis* **3**: 257-279 (1985).

3. Gamallo, C., et al., *Am. J. Pathol.* **142**: 987-993 (1993).

4. Groden, J., G. Joslyn, W. Samowitz, D. Jones, N. Bhattacharyya, L. Spirio, A. Thliveris, and et. al., *Cancer Res.* **55**:1531-1539 (1995).

5. Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens, and et. al., *Cell.* **66**: 589-600 (1991).

6. Taylor-Papadimitriou, J., and M. R. Stampfer., *Culture of Human Mammary Epithelial Cells. In Culture of Epithelial Cells.* Vol. 1. R. I. Freshney, editor. A John Wiley and Sons, Inc., New York. 107-133 (1992).

7. Shea, T.B, and M L Beerman, *BioTechniques* **10**: 288-294, (1991).

8. Sprott, S., K. Hammond, and N. Savage, *Anal. Biochem.* **194**:407-412 (1991).

9. Nickerson, J., G. Krockmalnic, and S. Penman, *Cell Biology: A Laboratory Handbook. In Cell Biology A Laboratory Handbook.* Vol. 1. J. E. Celis, editor. Academic Press, San Diego. 622-627 (1994).

10. Walen, K. H. and M. R. Stampfer, *Cancer Genet Cytogenet.* **37**: 249-61 (1989).

11. Nathke, I. S., C. L. Adams, P. Polakis, J. H. Sellin, and J. Nelson, *J. Cell Biol.* **134**:165-179 (1996).

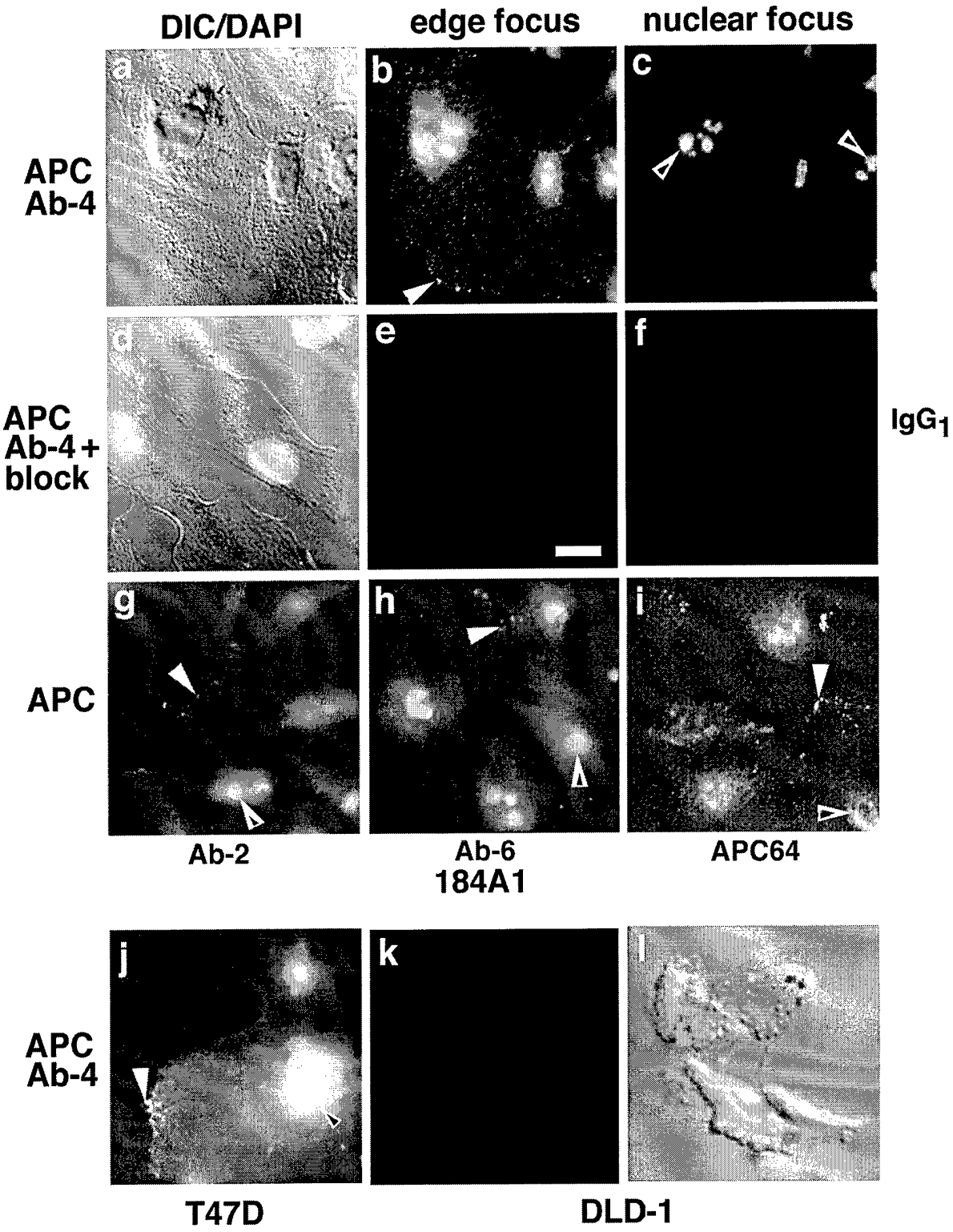
12. Powell, S. M., N. Zilz, B. Y. Beazer, T. M. Bryan, S. R. Hamilton, S. N. Thibodeau, B. Vogelstein and K. W. Kinzler, *Nature*. **359**: 235-7 (1992).

13. Robinson-Benion, C and J. T. Holt, *Methods in Enzymology* **254**:363-375 (1995).

APPENDIX

Figure 1. Localization of APC protein in 184A1 cells using immunofluorescence microscopy.

184A1 cells were grown on glass slides prior to fixation and immunofluorescence microscopy using Ab-4, an antibody specific for APC protein (b, c) or using Ab-4 preincubated with an APC peptide (e). (b) and (c) are photographs of the same group of cells taken at two focal distances to more clearly capture cell edge staining (b, solid arrow) and nuclear staining (c, open arrows). APC protein appears in a punctate pattern throughout the cytoplasm with areas of protein concentration at one edge (solid arrows). In addition, APC protein appears throughout the nuclei with a few areas of concentration (c). (a) and (d) are differential interference contrast (DIC) and DAPI views of the fluorescence views shown in (b and c), and (e), respectively. Controls include: f, 184A1 cells stained with nonspecific antibody IgG_1; g-i, 184A1 cells stained for APC using antibodies Ab-2 (g), Ab-6 (h) or APC64 (i); j, APC staining of breast cancer cell line T47D cells. For each antibody, both edge staining (solid arrows) and nuclear staining (open arrows) are apparent. In k and l, DLD-1 cells which express only truncated APC protein were stained using the C-terminal antibody Ab-4 to demonstrate staining specificity. Bar, 10 μm.


DIC/DAPI
edge focus
nuclear focus
a
b
c
APC
Ab-4
d
e
f
APC
Ab-4 +
block
IgG1
g
h
i
APC
Ab-2
Ab-6
184A1
APC64
j
k
l
APC
Ab-4
T47D
DLD-1

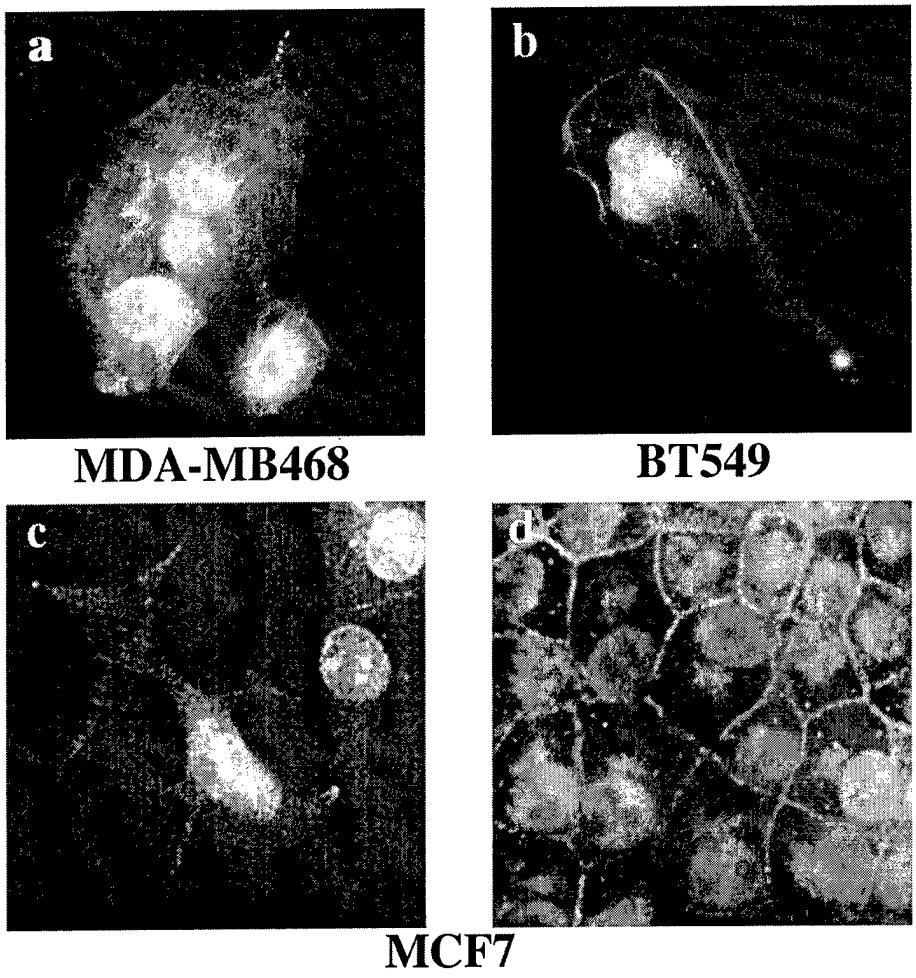


Figure 2. Localization of APC protein in cells from breast cancer cell lines.

Cells were grown on glass slides and stained with APC antibodies as described. The APC staining patterns of three breast cancer cell lines, not grown to confluence, MDA-MB468 (a), BT549 (b), and MCF-7 (c) are shown. As in 184A1 cells, the APC protein appears in a punctate pattern throughout the cytoplasm with areas of protein concentration at one edge, and also, throughout the nuclei with a few areas of concentration. In (d), MCF-7 cells were allowed to grow to confluence prior to staining, and display APC protein at cell-cell junctions.

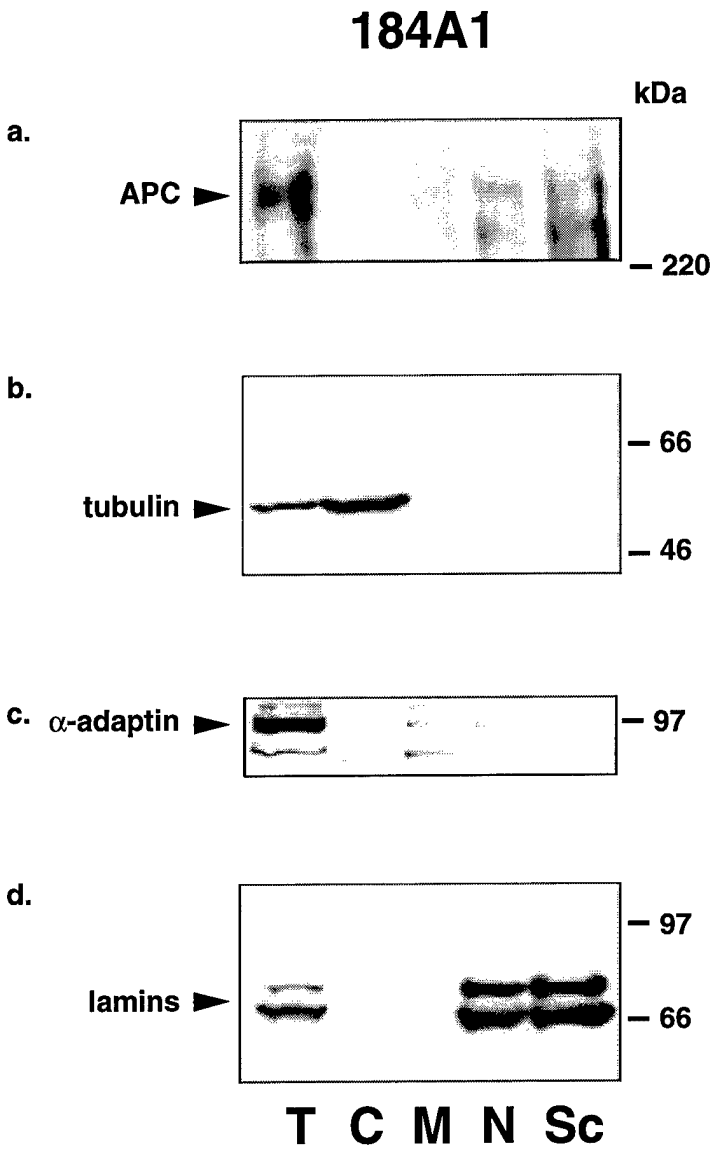


Figure 3. Full-length APC protein locates to both membrane/cytoskeletal and nuclear cell fractions in 184A1 cells.

Proteins within the various fractions of 184A1 cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Fractions are labeled at the figure bottom as follows: T (total), C (cytoplasm), M (membrane/cytoskeleton), N (nucleus), Sc (nuclear scaffold). The antibodies used for the Western immunoblots are as follows: panel a (APC), panel b (tubulin as a cytoskeletal marker), panel c (α-adaptin as a membrane marker), and panel d (lamins as nuclear and nuclear matrix scaffold markers).

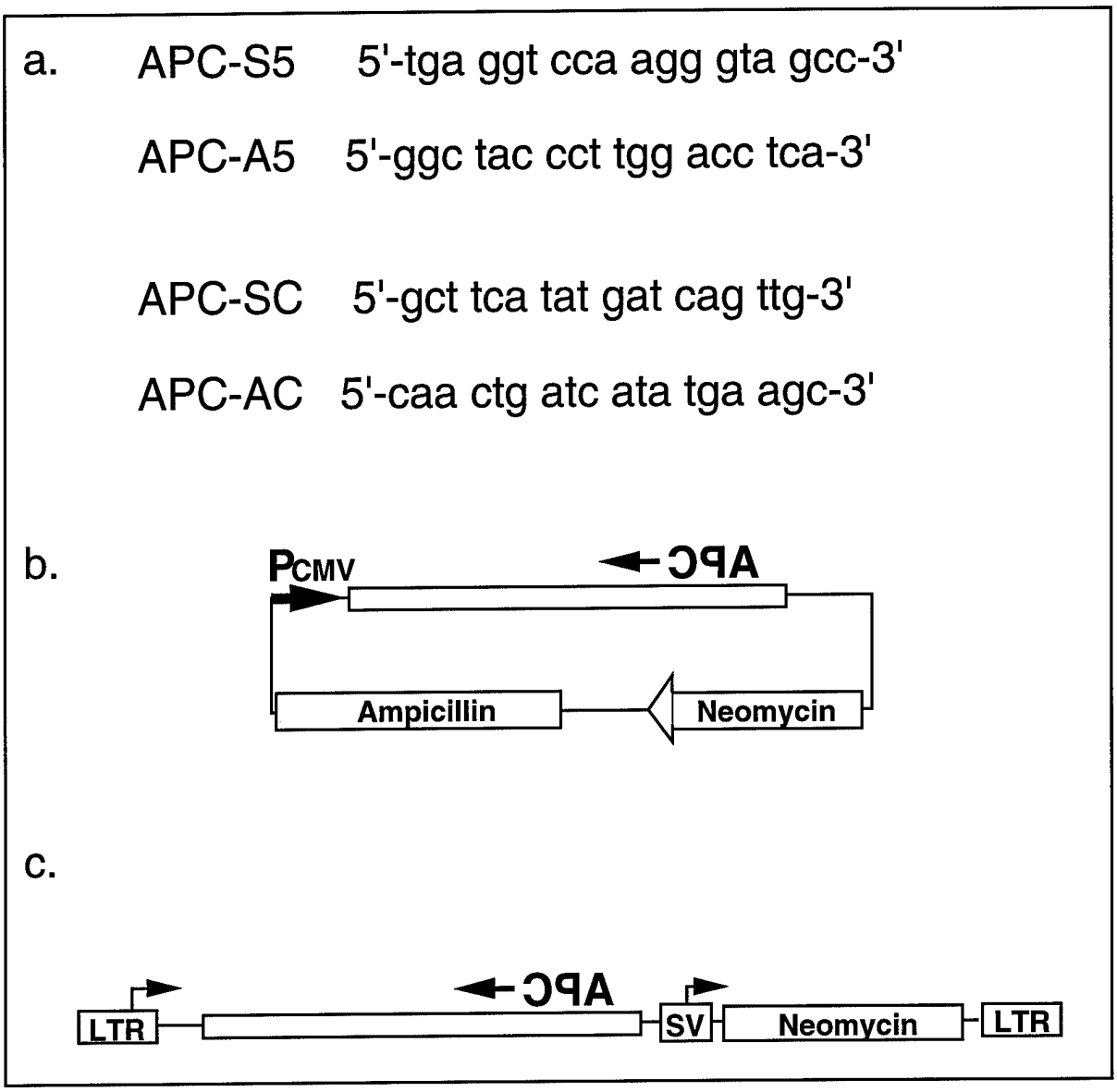


Figure 4. Schematic representation of constructs used for the antisense strategies.

a. DNA oligomers corresponding to sequences "antisense" to APC mRNA, either in the 5' untranslated region (APC-S5) or spanning the initiation codon (APC-AC) were sythesized and purified. "Sense" oligos (APC-S5 and APC-SC) were sythesized in parallel for use as negative controls.

b. Expression vector pCDNA3.1 containing the APC cDNA in reverse orientation. Other salient features include, the strong cytomegalo virus promotor (PCMV), the ampicillin resistance gene, and the neomycin resistance gene.

c. Retrovirus vector pLXSN containing the APC cDNA in reverse orientation. Other features include: long terminal repeat (LTR), Moloney murine promotor-enhanser; SV, simian virus 40 promotor; neomycin resistance gene.

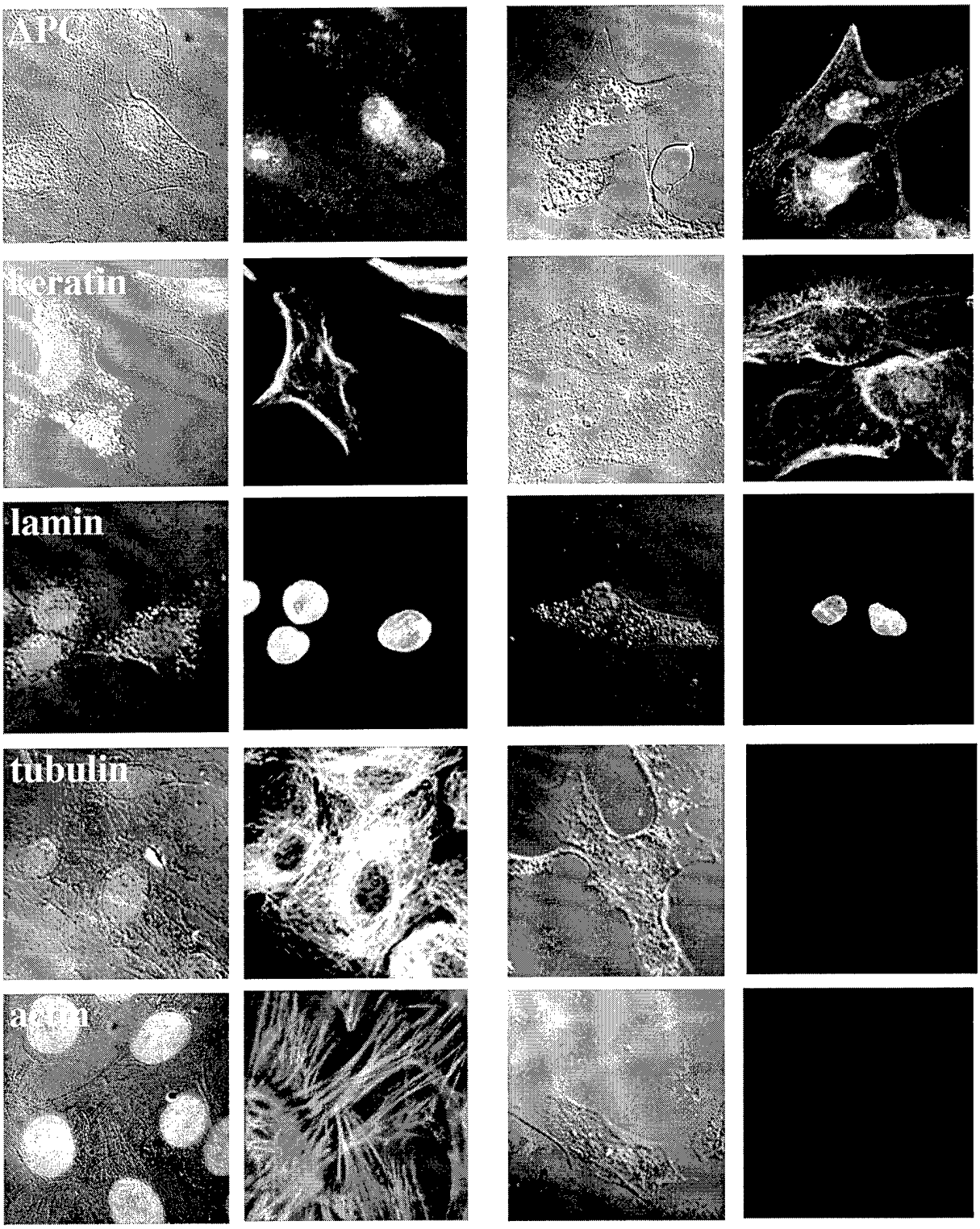
Figure 5 APC Protein distribution is altered following keratin destabilization with antibodies or with drugs that destabilize microtubule filaments.

184A1 cells were treated with drugs that destabilize microtubule filaments and then analyzed for distribution of various proteins by immunofluorescence microscopy. APC staining at the edge of untreated cells (a) was eliminated by treatment with colchicine (b), but not by γ-lumicolchicine (c) or cytochalasin B (d). Microtubule disruption as monitored by staining for α-tubulin was apparent in cells treated with colchicine (f), but not in cells treated with γ-lumicolchicine (g) or cytochalasin B (h), or in untreated cells (e). Actin distribution was unaltered in cells treated with colchicine (j) or γ-lumicolchicine (k) compared to untreated cells (i). Cells treated with actin destabilizing drug cytochalasin B showed a marked actin redistribution (l). Bar, 10 μm.

untreated
colchicine
lumi-colch.
cytochal. B
APC
tubulin
actin
a
b
c
d
e
f
g
h
i
j
k
l

Figure 6. APC protein remains with the intermediate filament proteins following sequential extraction.

184A1 cells, grown on glass slides, were sequentially extracted for all proteins not associated with the intermediate filament network and the nuclear scaffold. The left two columns represent untreated cells, whereas in the right two panels, cells were extracted. Panels are presented in pairs with the left panel of each pair corresponding to the DIC with DAPI overlay view of the cells stained in the right panel. Cells were immunostained for the following proteins as labeled: APC, keratin, lamin, tubulin, and actin. Proteins remaining cell-associated following treatment include APC, keratin, and lamins. Tubulin and actin were removed by the sequential extraction procedure. Bar, 10 μm.


APC
keratin
lamin
tubulin
actin